CD40-mediated activation of Ig-Cγ1- and Ig-Cε germ-line promoters involves multiple TRAF family proteins

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CD40 plays a critical role in immunoglobulin (Ig) class switching in B cells, but the molecular events involved remain poorly understood. Using CD40 mutants with impairments in their ability to bind selected TNF receptor-associated factor (TRAF) family proteins, we observed that CD40-mediated transcriptional induction of the germ-line Ig-Cγ1- and Ig-Cε promoters was markedly reduced by mutations that prevent TRAF2, TRAF3, TRAF5 or TRAF6 binding. Moreover, co-expression of trans-dominant inhibitory forms of TRAF2, 3, 5 or 6 with wild-type CD40 also suppressed induction of these promoters. Overexpression of TRAF2 or TRAF6 was sufficient to induce transcription of the C_H promoters through an NF-κB-dependent mechanism. In contrast, TRAF3 and TRAF5 failed to induce these promoters, implying a more indirect role for these TRAF family members. Altogether, the results demonstrate a non-redundant role for multiple TRAF in the signal transduction pathways by which CD40 induces transcription of germ-line C_H promoters. Since C_H germ-line transcription represents an obligatory step in Ig class switching in B cells, these findings suggest that interference with the functions of any of these TRAF might provide a means of preventing class switching for therapeutic purposes.

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1 Introduction

Upon exposure to T cell-dependent antigens, B cells undergo Ig class switching from membrane-bound IgM or IgD to production of IgG, IgA or IgE. The initial step required for initiation of class switching is expression of the respective unrearranged heavy chain constant region (C_H) gene, rendering these genes competent for the DNA recombination events responsible for Ig clas switching (reviewed [1]). According to the "accessibility" model for class switching, this change in C_H expression confers accessibility of the C_H locus to recombination factors [2, 3].

Targeted gene ablation experiments in mice and hereditary mutants in humans have demonstrated an essential role for CD40 in class switching [4–7]. CD40 is a member of the TNFR family that exerts its signaling capacity through interactions with TNFR family-associated factors (TRAF), namely TRAF2, 3, 5 and 6 (summarized in [8, 9]. Several unique binding motifs for TRAF have been identified within the cytosolic domain of CD40 and mutants have been generated which selectively bind certain TRAF but not others [8–11]. We investigated the influence of these CD40 mutations and the effects of overexpressing different TRAF on transcriptional induction of the Ig-Cγ1- and Ig-Cε germ-line promoters.

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Abbreviations: TRAF: TNF receptor-associated factors **WT:** Wild type **DN:** Dominant negative

2 Results

2.1 Correlation of TRAF binding sites in CD40 with induction of the Ig-Cγ1- and Ig-Cε germ-line promoters

We explored the ability of wild-type (WT) CD40 and various CD40 mutants with selective impairments in TRAF binding (Fig. 1 A) to transactivate luciferase reporter gene plasmids containing the CD40-responsive region (CD40RR) previously identified within the Ig-$C\gamma_1$ promoter and the complete Ig-Cε germ-line promoter [12, 13]. For these experiments, WT and mutant CD40 expression plasmids were transiently transfected with

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A

	WT	P227A	Q231A	Q234A	E235A	T254A	Q263A	Δ11aa	Δ15aa	Δ32aa	Δct
TRAF2	+++	+++	+++	+++	+++	-	+++	+	+	-	-
TRAF3	+++	+++	+++	+++	+++	-	-	+++	-	-	-
TRAF5(*)	+++	ND	ND	ND	ND	-	-	ND	ND	-	-
TRAF6(**)	+++	+++	+	+	-	ND	ND	ND	ND	+++	-

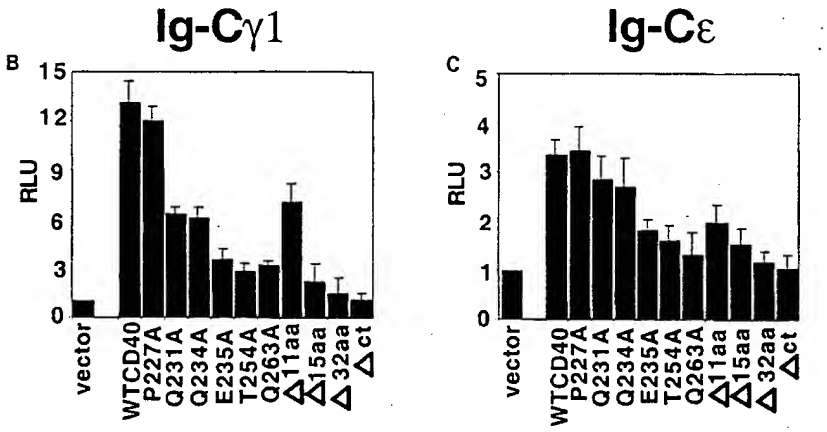

Figure 1. Effects of CD40 mutants on transactivation of IgCγ1- and Ig-Cε promoters. (A) TRAF binding characteristics of CD40 mutants is summarized for TRAF2, 3, 5 and 6, based on previous results [8, 9]. (B, C) Murine M12.4.1 B cells cultured in the presence of IL-4 were transiently co-transfected with 3.0 μg pcDNA3 control plasmid ("vector"), or with pcDNA3-human CD40 or various CD40 cytoplasmic domain mutants, as indicated, together with 0.5 μg luciferase reporter plasmids containing the Ig-Cγ1- (B) or Ig-Cε (C) germ-line transcript promoters and 0.5 μg pCMV-β-gal. Human CD40 was selectively activated by adding G28.5 antibody. Relative promoter activity (RLU) was assessed by luciferase assays, normalized for β-gal activity (mean ± SD; $n = 4$). (ND) not determined. (*) TRAF5 binds to CD40 only indirectly by heterodimerization with TRAF3 [8, 9]. (**) TRAF6 may bind to CD40ct indirectly through an adapter molecule [9].

the C_H region reporter plasmids into the B cell line M12.4.1, which is known to support Ig gene transcription [14, 15]. Though the low frequency of M12.4.1 cell transfection precluded direct assessment of expression, these CD40 mutants have been shown previously to be expressed at similar levels on the surface of a variety of highly transfectable cell lines and their altered interactions with TRAF have been confirmed in both lymphoid and non-lymphoid cells [8–11].

WT CD40 induced >12-fold and >3-fold increases in the activity of the Cγ1 and Cε reporter plasmids, respectively (Fig. 1). In contrast to WT CD40, all CD40 mutants with impaired ability to bind TRAF2, TRAF3, TRAF5 and/or TRAF6 displayed remarkably reduced activity in these assays. The CD40(Q263A) mutant which exhibits selective impairment in TRAF3 binding, for example, displayed only approximately 25 ± 10 % and 30 ± 10 % ($n = 3$) the activity of WT CD40 at inducing the Cγ1- and Cε promoters, respectively ($p < 0.01$; $p = 0.04$), providing indirect evidence of a requirement for TRAF3 for optimal CD40-mediated Cγ1- and Cε promoter transcription. Similarly, the CD40(Δ11aa) mutant that binds TRAF3 normally but exhibits significantly reduced binding to TRAF2 activated the Cγ1- and Cε promoters with only about half the potency of WTCD40 ($p = 0.03$; $p = 0.06$). A CD40 mutant (E235A) failing to associate with TRAF6 [10] also displayed drastic reductions in activation of the Cγ1- and Cε-promoters ($p = 0.02$; $p = 0.05$). Point mutations outside known TRAF interaction motifs did not influence activation of C_H promoters [*i.e.* CD40(P227A) and data not shown].

2.2 TRAF2 and TRAF6, but not TRAF3 and TRAF5 are independent activators of the Cγ1- and Cε promoters

In transient transfection assays where various TRAF were overexpressed in cells, both TRAF2 and TRAF6 independently activated the Cγ1- and Cε germ-line promoters (Fig. 2). Overexpression of TRAF6 resulted in a >70-fold induction of the Cγ1 promoter, whereas TRAF2 induced an approximately 6-fold increase (Fig. 2 A). In contrast, TRAF2 and TRAF6 induced comparable 4–5-fold increases in the transcription of the Cε promoter (Fig. 2 B). TRAF3 and TRAF5 did not activate the Ig-C_H reporter genes, suggesting an indirect mechanism for

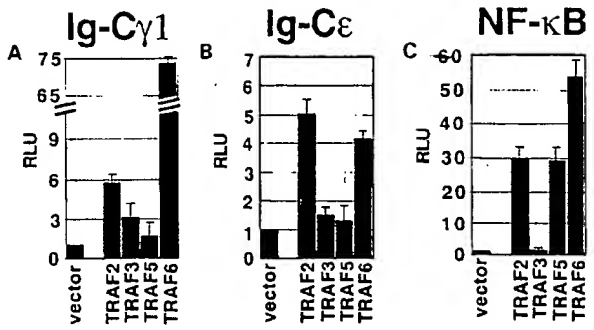

Figure 2. Overexpression of TRAF2 or TRAF6 induces Ig-Cγ1- and Ig-Cε promoters. Murine M12.4.1 B cells (A, B) or HEK 293N cells (C) were transiently transfected in 2.5 cm/6-well plates with 3.0 μg pcDNA3 control plasmid ("vector"), or expression plasmids encoding TRAF2, 3, 5 or 6 together with 0.5 μg luciferase reporter plasmids containing the Ig-Cγ1- (A) and Ig-Cε (B) germ-line transcript promoters and 0.5 μg pCMV-βgal, or with (C) 0.2 μg of the NF-κB reporter plasmid pUC13-4xNF-kb-luc and 0.2 μg pCMV-βgal. Relative promoter activation (RLU) was assessed by luciferase assays, and normalized by β-gal activity (mean ± SD; $n = 3$).

their involvement in CD40-mediated promoter activation, unlike TRAF2 and TRAF6. Overexpression of TRAF5 in HEK 293 cells, however, did result in increases in levels of NF-κB activity, similar to those generated by transfection of TRAF2 (Fig. 2C), verifying function of the TRAF5-encoding plasmid. As expected, TRAF3 overexpression did not induce NF-κB, thus confirming the specificity of these results.

2.3 CD40-mediated activation of the Cγ1- and Cε promoters is dependent upon NF-κB activation

Co-transfection of a plasmid encoding a dominant-negative (DN) IκB mutant [16] completely abolished CD40-mediated activation of the Cγ1- and Cε promoter constructs (Fig. 3). Overexpression of DN-IκB had the same effect on Cγ1- and Cε promoter induction by TRAF2 and TRAF6, suggesting that these TRAF also require the NF-κB pathway for their activation of the germ-line C_H promoters.

2.4 DN TRAF mutants suppress CD40-mediated activation of the Cγ1 and Cε promoters

Deletion of the N-terminal RING domain from TRAF family proteins has been shown to create transdominant inhibitory mutants that interfere with the functions of endogenous TRAF [17, 18]. Co-expression of DN

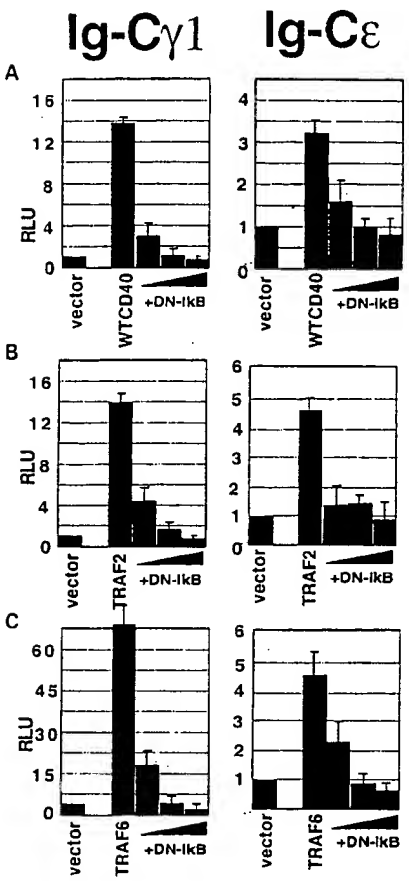

Figure 3. Inhibitory effect of DN IκB on CD40- and TRAF-mediated transactivation of Ig-Cγ1- and Ig-Cε promoters. (A) Murine M12.4.1 B cells were transiently cotransfected with 4 μg pcDNA3 control plasmid ("vector") or 3.0 μg pcDNA3 full-length CD40 and increasing amounts of DN IκB (0.1/0.3/0.9 μg), together with 0.5 μg luciferase reporter genes containing either the Ig-Cγ1- (left) or Ig-Cε (right) germ-line promoters and 0.5 μg pCMV-βgal. (B, C) Identical assays were performed using plasmids encoding TRAF2 or TRAF6 in place of CD40. In all cases, the total amount of transfected DNA was normalized by addition of empty pcDNA3. Relative promoter activity was assessed by luciferase assays and normalized by β-Gal activity (RLU) (mean ± SD; $n = 4$).

mutants of TRAF2, 3, 5 or 6 with WT CD40 in M12.4. cells suppressed CD40-mediated activation of both the Cγ1- and Cε promoters (Fig. 4). Though the ΔN-TRAF2 protein had only a moderate inhibitory effect, the ΔN-TRAF3, ΔN-TRAF5, and ΔN-TRAF6 mutants almost completely abolished CD40-mediated activation of the Cγ1- and Cε promoters. These results imply important roles for all four of these TRAF family proteins in C_H promoter transcription.

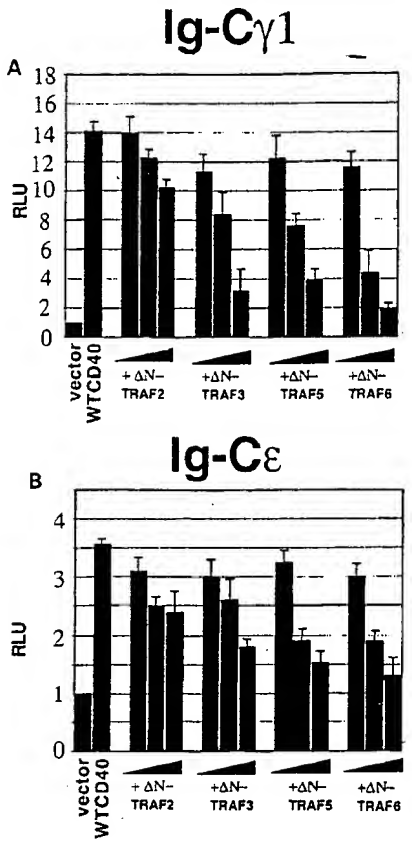

Figure 4. Influence of DN TRAF on CD40-mediated transactivation of Ig-Cγ1 and Ig-Cε promoters. (A) Murine M12.4.1 B cells cultured in IL-4-containing medium were transiently co-transfected with 5.25 μg pcDNA3 control plasmid ("vector") or 3 μg pcDNA3-human CD40 and increasing amounts of DN TRAF2, 3, 5, or 6 (0.25 μg/0.75 μg/2.25 μg) plasmids together with 0.5 μg luciferase reporter genes containing the Ig-Cγ1- (A) or Ig-Cε (B) germ-line promoters and 0.5 μg pCMV-β-gal. The human CD40 was selectively activated by using G28.5 antibody. Relative luciferase values were normalized to β-gal (RLU; mean ± SD; $n = 3$). Immunoblotting confirmed production of ΔN-TRAF proteins (not shown).

3 Discussion

The data presented here suggest a requirement for all TRAF molecules known to associate with CD40 for optimal induction of Ig-Cγ1- and Ig-Cε promoter transcription. Mutations of TRAF binding motifs within the CD40 cytoplasmic tail or overexpression of DN TRAF mutants led to significant decreases in CD40-mediated activation of reporter genes containing germ-line promoters for Ig-Cγ1 and Ig-Cε. Previous investigations of the requirements for various TRAF on CD40-mediated NF-κB induction and JNK activation have demonstrated redundancy among TRAF (reviewed in [9]). In contrast, studies of Cγ1- and Ig-Cε promoter induction imply a non-redundant role for several TRAF for transcriptional activation of these promoters involved in Ig gene expression and control of class switch recombination. Requirements for combinations of multiple TRAF may help to ensure B lineage-specific induction of C_H promoter transcription.

TRAF2, TRAF5 and TRAF6 are all known to activate NF-κB through their ability to bind activators of the IκB kinase complex such as NIK and RIP-2, and MEKK1. Previously, CD40-induced NF-κB/p50-RelA and NF-kB/p50-Rel-B dimers were shown to be important for transactivating the Ig-Cγ1 promoter [12]. In contrast, CD40-mediated induction of the Ig-Cε promoter has been associated with NF-κB/p50-RelC dimers, which in combination with active STAT6 induced by IL-4 or IL-13 appear to be essential for transactivation of this C_H promoter [15]. These differences in Rel protein patterns may depend upon either participation of other co-stimulatory factors besides TRAF or on differences in the types of NF-κB/IκB complexes which specific TRAF preferentially activate. However, while experiments with DN-IκB indicate that NF-κB is necessary for CD40- and TRAF-mediated induction of the Cγ1- and Cε promoters, this does not exclude the participation of other types of transcription factors, such as STAT, which are activated by the class switch supporting cytokine IL-4 [19].

In contrast to TRAF2, 5 and 6, the TRAF3 protein does not activate NF-κB directly and may instead rely on NF-κB-independent mechanisms for transducing signals. The findings that (1) CD40 mutants which lack TRAF3 binding capability displayed impaired induction of Ig-Cγ1- and Ig-Cε promoter transcription and that (2) ΔN-TRAF3 overexpression suppressed CD40-mediated induction of C_H promoters suggests a critical role for TRAF3 in germ-line promoter activation. The modulation of C_H promoter transactivation by TRAF3, however, may be an indirect consequence of its requirement for recruitment of TRF5 to CD40 receptor complexes via formation of TRAF3/TRAF5 hetero-oligomers [8, 9]. This may also explain why overexpression of TRAF5 by itself was insufficient to transactivate the Ig-Cγ1- and Ig-Cε promoters. Though a recent report suggested that overexpression of TRAF3 may suppress rather than enhance Ig production induced by CD40, the effect of TRAF3 on Ig class switching was not examined [20]. Future studies, particularly experiments exploiting transgenic and gene knockout mice, will help to further elucidate the *in vivo* importance of specific TRAF family proteins for regulation of Ig class switching in B cells.

4 Materials and methods

4.1 Plasmids

Plasmids have been described elsewhere [9, 12, 13] which contain cDNA encompassing the complete open reading frames of CD40 (pcDNA3-CD40/mutants), huTRAF2-[pcDNA3-HA-TRAF2 and pcDNA3-HA-TRAF2 (residues 248–501)], huTRAF3b [pcDNA3-HA-TRAF3b, pcDNA3-HA-TRAF3b (residues 355–587)], huTRAF-5 (pMKIT-HA-TRAF5) and muTRAF5 [pcDNA3-FLAG-TRAF5 (residues 294–558)], huTRAF6 [pcDNA3-myc-TRAF6 and pcDNA3-myc-TRAF6 (residues 301–530)], the promoter-containing reporter gene plasmids pUC13-4xNF-κB-Luc (containing four tandem HIV-NF-κB response elements and the minimal fos promoter) and pFL-CD40RR-Luc (containing the mouse minimal fos-promoter and the –99 to –43 CD40-responsive element of the germ-line Cγ1 promoter), and pFL-163ε-Luc (containing mouse minimal fos-promoter and the –163 to +53 CD40-responsive element of the germ-line Cε promoter).

4.2 Cell culture and transfections

293N cells were obtained from American Type Culture Collection (ATCC; Rockville, MD) and cultured in DMEM-high glucose media (Gibco-BRL) supplemented with 10% FBS (Hyclone), 1 mM L-glutamine and antibiotics. The murine B cell line M12.4.1 [21] was maintained in RPMI (Gibco-BRL) containing the same additives plus 1 mM sodium pyruvate, MEM essential amino acids (Gibco-BRL) and 50 nM 2-ME.

4.3 Reporter gene asssay

For Ig class switch reporter gene assays, M12.4.1 cells were seeded at 4×10^5–5×10^5 cells/ml in 6-well plates and transfected with various expression plasmids by lipofection (Lipofectamine Plus, Gibco), then stimulated 12 h later with G28.5 anti-human CD40 Ab (ATCC, 10 μg/ml) and murine IL-4 (Genzyme, 10000 U/ml) for 12–18 h. Cell lysates were then evaluated for luciferase and β-galactosidase (β-gal) activity.

For NF-κB reporter gene assays, 293N cells were transfected using SuperFect (Qiagen) with 1 μg (total) plasmid DNA at 60–80% confluency in 12-well plates. After 1.5 days, cells were lysed in 0.1 ml Promega-lysis buffer. Lysates were measured for luciferase activity using a luminometer (EG&G Berthold) and Promega luciferase kits, normalizing relative to β-gal.

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